

The basis of high resolution separation of small DNAs by asymmetric-voltage field inversion electrophoresis and its application to DNA sequencing gels

Bruce W. Birren*, Melvin I. Simon and Eric Lai¹

Division of Biology, California Institute of Technology, Pasadena, CA 91125 and ¹Department of Pharmacology, The University of North Carolina, Chapel Hill, NC 27514, USA

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ABSTRACT

We have previously shown that asymmetric-voltage field inversion electrophoresis produces more uniform separation for fragments between 1 and 50 kilobases (kb) than other modes of pulsed field gel electrophoresis. We now report on the basis of this phenomenon. As in conventional electrophoresis, the pulsed field mobility of DNAs between 1 and 50 kb varies with voltage in a size dependent manner. The complex migration pattern obtained with asymmetric-voltage field inversion electrophoresis reflects the difference between the mobilities of each sized fragment under the conditions used for the forward and reverse fields. We have applied this technique to DNA sequencing gels and find improvement in resolution for single-stranded fragments in polyacrylamide gels.

INTRODUCTION

By using low voltage gradients and low concentration agarose gels, conventional electrophoresis can resolve DNA fragments up to 50 kb (1,2). Most common agarose gels however, do not separate fragments much larger than 15–20 kb. Electrophoresis of single stranded DNA in 80 cm long denaturing gels of 6% polyacrylamide permits single base resolution of DNA fragments to about 500 bases per loading. Information is lost with both agarose and polyacrylamide electrophoresis due to the fact that the migration of DNA fragments in conventional electrophoresis depends on the logarithm of their size and consequently, resolution diminishes dramatically as the fragment size increases. Further, above a specific size limit for each gel, DNA molecules move with a uniform mobility independent of further increases in size (3). By subjecting DNA to alternating (pulsed) electric fields of different orientations, Schwartz and Cantor were able to extend the useful size range for separation of DNA by gel electrophoresis (4). In addition, these large DNAs (greater than 50 kb) separated with pulsed fields migrate with an nearly linear relationship between their size and distance (5–9). The particular size range separated in a pulsed field gel (PFG) is governed by the interval between field switching, with increasingly larger DNAs being resolved with increasingly long switch intervals

(5–7,9,10). With short switch intervals, pulsed field gels are useful for the separation of smaller DNA, for example in restriction mapping of cosmid clones (11).

Multiple techniques have been developed for switching electric field orientations during electrophoresis. The simplest involves periodic reversal of the field, termed field inversion gel electrophoresis (FIGE-ref.12). Net migration is achieved by employing forward and reverse fields which are asymmetric with regard to either voltage or duration (12). Other forms of pulsed field electrophoresis employ alternating fields which differ in orientation by angles other than 180°, for example 120° (13). We have previously reported that asymmetric-voltage field inversion gel electrophoresis produces more uniform separation for DNA fragments between 1 and 50 kb than other modes of PFG electrophoresis (14). We now present evidence concerning the basis for the differences in migration observed when 1 to 50 kb fragments are separated using the different modes of pulsed electric fields. We have applied these results to DNA sequencing gels.

MATERIALS AND METHODS

DNA Samples and Gels

Samples for the agarose gels were the 1 kb ladder and High Molecular Weight markers of BRL (Gaithersburg MD) which are loaded as a liquid sample. Dilutions, where necessary, were made in TE (10 mM Tris, pH 8.0, 1 mM EDTA). All gels were 1% SeaKem LE agarose (FMC BioProducts, Rockland, ME), cast and run in 0.5×TBE (1×TBE is 90 mM Tris, 90 mM Borate, 2 mM EDTA (15)).

Electrophoresis and Determination of Relative Mobility

All gels were run with the PACE electrophoresis system (16) using two alternating homogeneous fields and constant reorientation angles. Gel conditions and photography were as described (9). Gels were run at 14°C in 0.5×TBE. The distance migrated was measured from photographic negatives and used to calculate the pulsed field mobility (cm/hr). The migration of all fragments is expressed relative to that of the 1018 bp

* To whom correspondence should be addressed

component of the 1 kb ladder. The mobility of the 1018 bp fragment is given in the figure legends.

The two-dimensional gels of figure 2 were run by programming the 120° reorientation separation and the field inversion separation to be run consecutively. First two alternating fields of 0.8 second duration at 6 V/cm oriented at either 120° or 240° were run for 10 hours (0° being defined as upwards, towards the wells, and sweeping clockwise as described in ref. 16). Then a 14 hour run alternated between a 'forward' 9 V/cm field oriented at 90° and the 'reverse' 6 V/cm field of 270° orientation, each of 0.8 sec duration.

The gels of figure 3 which used 120° alternating fields of different voltages were run using a repeating cycle of 12 fields. The duration of each field were 0.8 seconds. The voltages and orientations of these fields were 1; 9 V/cm, 120°. 2; 6 V/cm, 240°. 3; 9 V/cm, 120°. 4; 6 V/cm, 240°. 5; 9 V/cm, 120°. 6; 6 V/cm, 240°. 7; 6 V/cm, 120°. 8; 9 V/cm, 240°. 9; 6 V/cm, 120°. 10; 9 V/cm, 240°. 11; 6 V/cm, 120°. 12; 9 V/cm, 240°. Alternatively, 3 V/cm fields were substituted for the 6 V/cm fields in the sequence described above.

The gel described in figure 4B, in which separate 120° pulsed field configurations were used in both the forward and reverse orientation was run by programming 6 sequential blocks of pulsing conditions, with each block consisting of two alternating electric fields differing in orientation by 120°. The orientation, duration, and voltage gradient used for each of the 6 blocks were: Block I; forward, 4 hr., 9 V/cm. Block II; reverse, 4 hr., 6 V/cm. Block III; forward, 4 hr., 9 V/cm. Block IV; reverse, 4 hr., 6 V/cm. Block V; forward, 2 hr., 9 V/cm. Block VI; reverse, 2 hr., 6 V/cm. The forward blocks consisted of two alternating electric fields oriented at either 120° or 240° relative to the top of the gel. The reverse blocks consisted of two alternating fields oriented at either 60° or 300°. Pulse intervals for all of the fields used for this gel were 0.8 sec.

Field inversion sequencing gels

Radio-labelled products of di-deoxy sequencing reactions of bacteriophage M13 mp18 were run in 6% acrylamide gels on an 80 cm apparatus (BioRad Laboratories, Richmond CA) using 1×TBE. Gels were pre-run to attain the running temperature prior to sample loading. Conventional sequencing gels were run for 4 hr. at 2800 V. Both the conventional and the field inversion gel were run until the bromphenol blue marker was at the bottom of the gel. Asymmetric voltage field inversion gels were run using a modified HU3000 switching unit (IBI) and LKB power supplies. The HU3000 switching unit has been altered so that two separate power supplies are connected to the switching unit and are alternately connected to the gel. Following electrophoresis, gels were dried and exposed to X-ray film, and mobilities were measured from the bands on the autoradiographs.

RESULTS

Improved Resolution of 1–50 kb Fragments by FIGE Using Different Forward and Reverse Voltages

Unlike pulsed field separations of larger DNAs (5,6,7,9), fragments of the size range from 1 kb to 50 kb do not migrate as a linear function of their size. Figure 1a shows the separation of 1–50 kb DNA fragments in 1% agarose using two alternating fields of 6 V/cm (0.8 sec duration) which differ in their orientation by 120°. The spacing between the elements of the 1 kb ladder is not even, but is greatest between the smaller sized fragments

and decreases with increasing fragment size. Thus, the distance separating the 2 kb and 3 kb bands is equivalent to that between the 15 kb and 48.5 kb bands of the high molecular weight marker. The 38.4 kb and the 48.5 kb bands are not separated using these conditions. The use of longer switch intervals increases the size range of molecules separated in pulsed field gels (4). Figure 1b shows that while increasing the switch interval to 4 seconds leads to separation of the 38.4 and 48.5 kb bands, resolution has diminished for bands in the range of 12 to 33 kb. The spacing of the fragments between 1 and 12 kb is virtually unchanged from the 0.8 sec gel of figure 1a. Thus lengthening the switch interval used with 120° pulsed field gels does not improve the ability to separate fragments over the entire size range of these markers. The PACE electrophoresis system permits direct comparison of different modes of pulsed field electrophoresis, such as 120° PFG and field inversion, in the same gel box (16). We have previously reported that field inversion gel electrophoresis (FIGE) which makes use of fields of uniform duration and dissimilar voltage produce fundamentally different mobilities for fragments between 1 and 50 kb than other pulsed field methods (4). Figure 1c shows a field inversion separation of these same fragments which was carried out using a 9 V/cm forward field and a 6 V/cm reverse field (each of 0.8 sec duration). These conditions yield a more uniform separation over the entire size range from 1 to 50 kb, with notably improved resolution between the larger molecules.

The relative pulsed field mobility of the DNA fragments in these gels is plotted in figure 1d. Note that these curves employ a logarithmic scale for the size, as is customary with conventional electrophoresis (3) and in contrast to plots from pulsed field gels with larger DNAs. The curves representing both the 0.8 and the 4 sec gels are bi-phasic, with the portion reflecting molecules from 1 to 5 kb being identical and following a nearly straight line. Above this size range, the two curves follow generally straight lines, but of different slopes. The highest resolution obtained with these gels is found in the size range of 1 to 5 kb. Figure 1d makes it clear that resolution for the larger fragments (15 to 50 kb) is vastly improved in the field inversion gel compared to the 120° pulsed field gels of figures 1a and 1b. In fact, the relative mobility of fragments separated by asymmetric-voltage field inversion shows a nearly linear relationship with size over the range of about 15 to 50 kb. For fragments between 6 and 12 kb, the resolution is identical between the gels shown in Figure 1a and 1c. Resolution is diminished for the smallest fragments on the gel when the separation occurs by field inversion with different voltages. However, this is the region in which the separation is the greatest and thus in which any diminution in resolution is least critical.

The Order of Migration Reflects Fragment Size Using These Field Inversion Conditions

It is known that pulsed field electrophoresis can induce fragment migration in an order which does not reflect increasing size. This was demonstrated initially by Maynard Olson and colleagues for both field inversion (5) and other modes of pulsed field electrophoresis (15). Molecular weight markers may not be assumed to migrate in a strict order based upon size when new electrophoresis conditions are employed. Two-dimensional electrophoresis was used to verify the correspondence between size and migration order plotted in figure 1d. Each of the two sets of markers were first run using the 120° pulsed field conditions of figure 1a. The fragments were then separated with the asymmetric-voltage field inversion conditions of figure 1c,

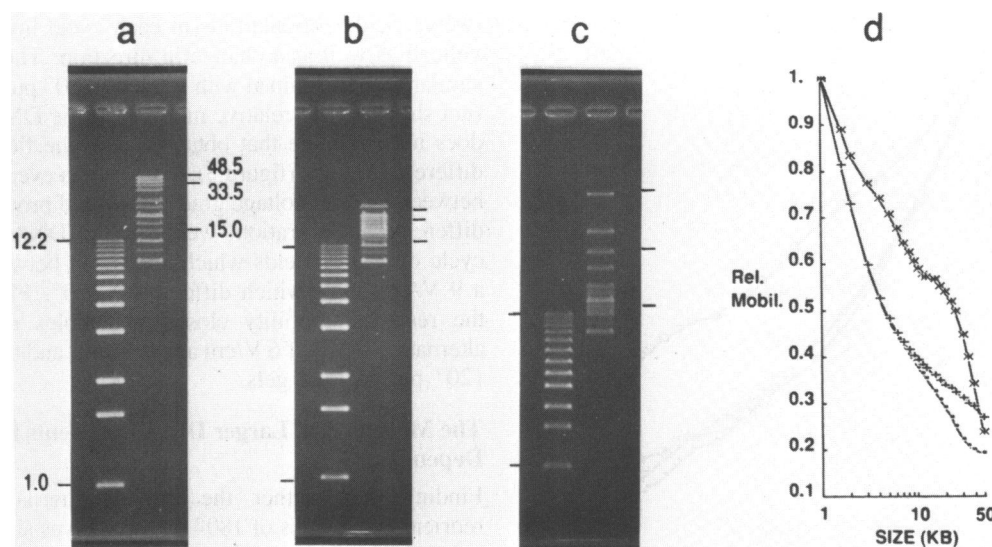


Figure 1. Comparison of 120 degree pulsed field and field inversion electrophoresis using different voltage gradients to separate DNAs from 1 to 50 kb. DNAs were separated in 13 cm square 1% agarose gels, and stained with ethidium bromide. The entire length of the gel is shown in this and all subsequent figures. a) 120 degree pulsed field electrophoresis was carried out using a switch interval of 0.8 s with alternating fields of 6 V/cm, and a run duration of 10 hrs. b) Conditions were identical to those in a) except that the switch interval was 4 s. c) Field inversion electrophoresis was carried out using a forward field of 9 V/cm and a reverse field of 6 V/cm, each of 0.8 s duration. Total run time was 16 hrs. d) The mobility of all fragments seen in a (.), b (+), and c (*), was plotted relative to that of the 1 kb band in each gel. The mobility of the 1 kb fragment in each of the gels was: a) 1.05 cm/hr; b) 1.04 cm/hr; c) 0.616 cm/hr

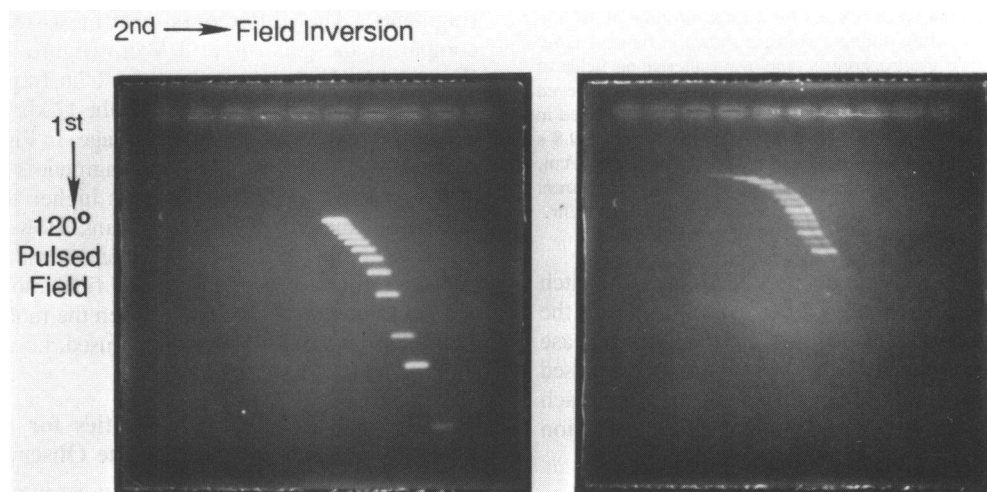


Figure 2. Two dimensional separation using 120° pulsed fields and asymmetric-voltage field inversion. Samples were loaded in the second lane from the left of 1% gels. The samples were run first for ten hr using two alternating fields of 6 V/cm which differed in orientation by 120°. The samples were then run with field inversion conditions perpendicular to the first separation using a forward field of 9 V/cm and a reverse field of 6 V/cm for 14 hr. A switch interval of 0.8 sec was used for both dimensions. Left—1 kb ladder. Right—High Molecular Weight markers.

in a direction perpendicular to the initial separation. The resulting gels are shown in figure 2. The same correspondence between mobility and size is found for fragments from 500 bp to 48.5 kb using each of these two different conditions. In addition, figure 2 provides a clear visual demonstration of the differences in mobility between the two electrophoresis techniques. The bands between 5 and 12 kb all migrate on a diagonal, indicating similar mobilities with each of the two different modes of electrophoresis. The fragments from 1 to 3 kb run significantly slower in the field inversion dimension than would be expected based upon the migration of the larger fragments. The largest fragments (19.4–48.5 kb) are separated to a much greater degree by the field inversion conditions than with 120° pulsed fields. It is these

two features, the compression of the smallest fragments and the greater separation between the large fragments that makes asymmetric-voltage field inversion so effective for separating fragments in this size range. Further work was undertaken to explore the reasons for this effect.

Symmetric-voltage FIGE Does Not Give Rise to Extended Resolution

The mobility obtained with the asymmetric-voltage field inversion gels could be due to physical properties arising from the 180° reorientation required of molecules undergoing field inversion electrophoresis. DNAs were separated by FIGE using a constant voltage gradient of 6 V/cm. The forward and reverse switch

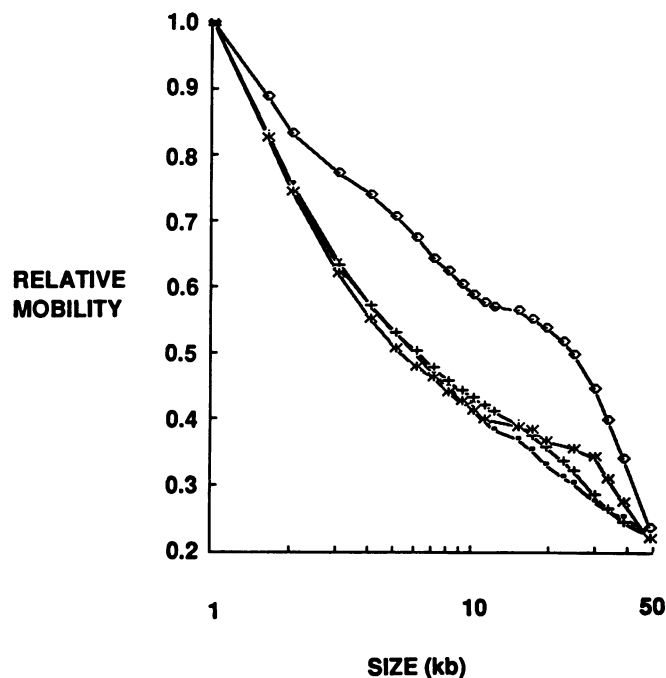


Figure 3. The relative mobility of fragments separated with various patterns of alternating fields of different voltages. Constant field-strength field inversion electrophoresis (*) was carried out at 6 V/cm with a forward-field duration of 1.2 sec and a reverse-field duration of 0.8 sec for a total run time of 16 hrs. The field inversion separation using different voltages shown in figure 1 is re-plotted here (O). Pulsed field electrophoresis employing alternating fields of different strengths and which differed in orientation by 120 degree was achieved by programming a repeating cycle of 12 different voltage states as described in Materials and Methods. This was carried out with alternating fields of 0.8 s duration, and field strengths and electrophoresis runs of 9 V/cm and 6 V/cm, 6.5 hrs (+), or of 9 V/cm and 3 V/cm, 9 hrs (.). The mobility of the 1 kb fragment in each of the gels was: (+), 1.45 cm/hr; (.), 1.15 cm/hr; (*), 0.41 cm/hr.

intervals were chosen to yield a product of the voltage \times switch interval that was identical to those of figure 1c. The plot of the relative mobility of this gel in Figure 3 shows a slight increase in the mobility of the large fragments compared to the 120° pulsed field gels. However, the resulting resolution does not approach that seen when different voltages were used for field inversion electrophoresis.

Voltage Alternations are Not Sufficient to Produce the Mobility Shift

It is possible that the difference in migration of these fragments between asymmetric-voltage FIGE and other pulsed field techniques is due to an additional physical aspect of separation which arises with alternating fields of different voltage. For example, DNA molecules extend to different lengths when exposed to voltage gradients of varied strength, and therefore require different lengths of time to relax (17). This could be important as some amount of relaxation apparently occurs prior to migration of DNA in the new field direction (18). In addition, voltage dependent changes in mobility have been demonstrated for conventional (3,19) as well as pulsed field (20) gels. We investigated the effects of alternations in field strength by using a programmed regimen of alternating electric fields of 9 V/cm and 6 V/cm, each of which differed by 120°. These are the same field strengths used in the field inversion gel shown in figure 1c. To maintain straight running lanes, a symmetric cycle of twelve fields was used (see Materials and Methods). Ten of the

twelve field reorientations in each cycle involved a change in voltage as well as a change in direction. The resulting gel was similar to that obtained with standard 120° pulsed field switching (not shown). The relative mobility of the DNA from such a gel does not resemble that obtained with the field inversion using different voltages (figure 3). To see if an even greater difference between the two voltage gradients would produce any detectable difference in migration, we separated DNA using a repeating cycle of twelve fields which alternated between a 3 V/cm and a 9 V/cm field, which differed by 120°. Figure 3 shows that the resulting mobility closely resembles that obtained with alternating fields of 6 V/cm and 9 V/cm, and the constant 6 V/cm 120° pulsed field gels.

The Migration of Larger DNA Fragments is Strongly Voltage Dependent

Finding that neither the use of alternating voltages nor reorientation angles of 180° by themselves significantly alter the pulsed field mobility of these fragments, we examined more closely the relationship between voltage and migration for 1 kb to 50 kb DNAs separated using 120° pulsed fields. Figure 4 shows the migration of DNA fragments using voltage gradients of 3 V/cm, 4.5 V/cm, 6 V/cm, and 9 V/cm. The switch intervals used for each e chosen to maintain a constant product of the switch interval \times voltage for all of these conditions. With increasing voltage there is a progressive increase in the mobility of the larger fragments. The differences are most readily apparent by comparing the gels run at 3 V/cm (figure 4a) and at 9 V/cm, (Figure 4d). In these two gels the 1 kb fragment has migrated the same distance down the gel, yet the 12 kb fragment has moved nearly twice as far at the higher voltage. In Figure 4e the mobility of the DNAs relative to the 1 kb fragment is plotted. Resolution of the smallest DNA fragments is higher for the low voltage separations than the high voltage runs. However, for fragments above 10 kb, resolution steadily increases with increasing voltage. Similar differences among pulsed field mobilities obtained at different voltages are obtained when the mobility is normalized to account for the different voltages used, i.e., plots of cm/hr/volt (not shown).

The Difference Between Mobilities for the Forward and Reverse Voltage Accounts for the Observed Migration

The observation that these DNAs move in a voltage dependent manner suggested that the difference in the mobility of molecules separated using asymmetric-voltage FIGE could result from differences between the pulsed field mobility of the DNAs migrating with the forward and reverse voltage gradients used. To test this hypothesis, DNAs were separated using standard 120° pulsed field gels with voltage gradients of either 6 or 9 V/cm (0.8 sec switch interval). Photographs of the gels are shown in figure 5A. The relative mobility of the fragments are plotted to the right of the gels. The plots of relative mobility show the greatest difference lies with the fragments larger than about 8 kb. We have calculated the mobility of each band under the two different voltage conditions. Subtracting the values obtained for the 6 V/cm gel (the gradient used for the reverse field of Figure 1c) from those of the 9 V/cm gel (the forward field) gives values reflecting the difference in migration between the two different voltages for the whole range of fragments. In figure 5A we have plotted these calculated values along with the observed mobilities for the 9 V/cm and 6 V/cm field inversion separation. There is extraordinary agreement between these two curves, both in the

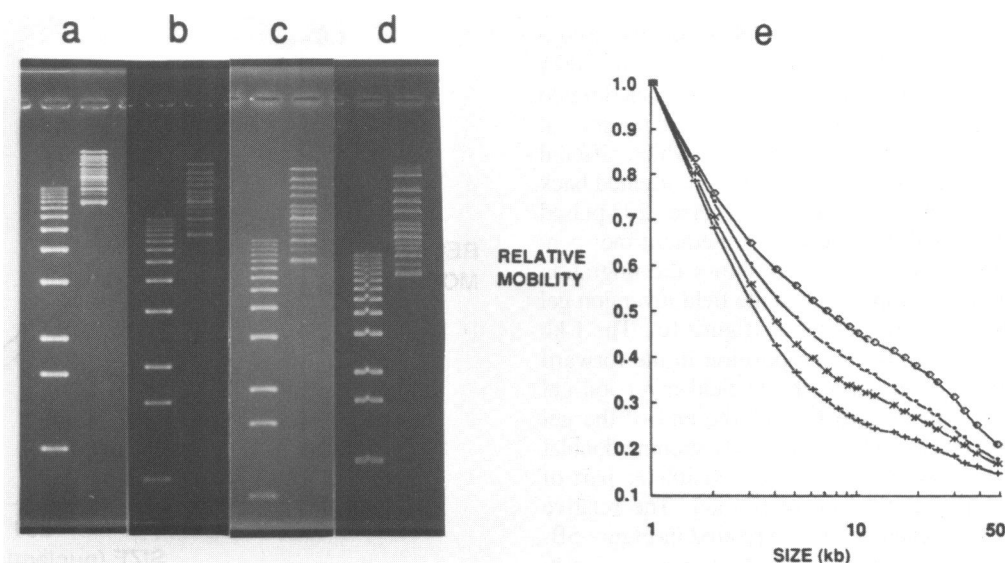


Figure 4. 120° pulsed field gels which keep a constant product of voltage times switch interval. The stained gels are shown for separations using standard 120° pulsed fields of the following field strength, switch interval, and run duration: a) 3 V/cm, 2.4 sec, 20 hr., b) 4.5 V/cm, 1.6 sec, 13 hr. c) 6 V/cm, 1.2 sec, 10 hr. d) 9 V/cm, 0.8 sec, 5.5 hr. e) The mobility of fragments in these gels is plotted relative to the 1 kb band. (+) 3 V/cm 2.4 sec, (*) 4.5 V/cm 1.6 sec, (.) 6 V/cm 1.2 sec, (○) 9 V/cm 0.8 sec. The mobility of the 1 kb fragment in each of the gels was: a, 0.49 cm/hr; b, 0.82 cm/hr; c, 1.12 cm/hr; d, 1.86 cm/hr.

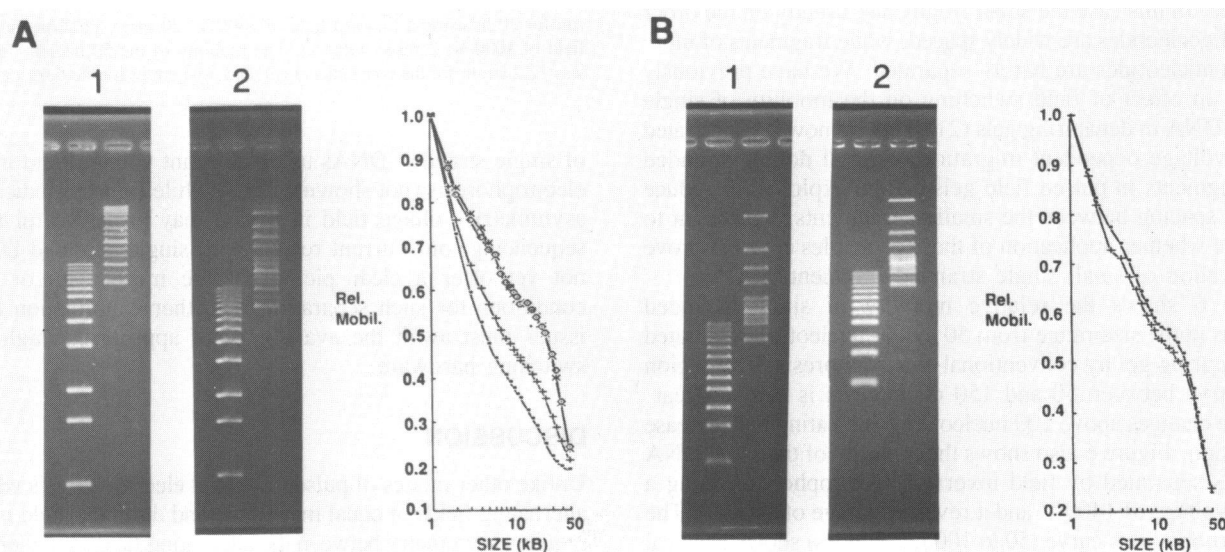


Figure 5. A Differences in mobility for fragments separated using pulsed fields of 9 V/cm and 6 V/cm. 120° pulsed field gels were run with a switch interval of 0.8 sec and field strengths of either 1) 6 V/cm (10 hr run) or 2) 9 V/cm (6 hr run). The relative mobility of each of these separations is plotted to the right of the photographs, (.) 6 V/cm, (+) 9 V/cm. For comparison, the mobility of the field inversion gel shown in figure 1 is replotted (*). The pulsed field mobility (cm/hr) of each of the bands observed at 6 V/cm was calculated and subtracted from the mobility of the same sized fragments at 9 V/cm. The resulting mobilities are also plotted relative to that of the 1 kb fragment (○). B The stained gel of Figure 1c, representing separation by field inversion electrophoresis with different voltages 1), is shown next to a gel which employed a programmed regimen of 120° pulsed fields of 0.8 sec duration which were oriented alternately forward or backward with respect to the wells 2). Alternating cycles of 120° pulsed field electrophoresis were used, in which both of the forward fields were 9 V/cm, and both reverse fields were 6 V/cm (see Materials and Methods). The mobility of DNAs from these gels are plotted relative to the 1 kb fragment. (.) field inversion (9 V/cm forward, 6 V/cm reverse), (+) alternating forward and reverse 120° pulsed field. The mobility of the 1 kb fragment in each of the gels was: Panel A 1) 1.05 cm/hr; 2) 1.72 cm/hr; Panel B 1) .616 cm/hr; B 2) 0.741 cm/hr.

initial and final slopes and the occurrence of the 'hump' at about 12 kb. The difference in mobility between the two voltages is greatest for the large fragments. Thus, the large fragments move relatively faster under the influence of the forward 9 V/cm field than they do under the influence of the 6 V/cm reverse field, while the smaller fragments migrate at more similar rates at the different voltages.

The mobility of DNA in the field inversion gels of dissimilar voltages may simply result from the differences in the migration at the two different voltages and may not reflect a property inherent to field inversion electrophoresis. As a further test we separated DNA using pulsed fields of different voltage to move the DNA 'down' the gel (away from the wells), and then back 'up' towards the wells, but which did not use the 180°

reorientation of field inversion. To accomplish this we used a programmed regimen which for several hours moved the DNA down the gel using two alternating fields of 9 V/cm which differed in orientation by 120°. The DNA was then subjected for several hours to two alternating electric fields of 6 V/cm which differed in orientation by 120°, but with the net migration oriented back towards the wells. This cycle of forward and reverse 120° pulsed field migration was repeated three times, with equal time spent going forward and backward. Figure 5B shows the migration obtained by this switching compared with the field inversion gel of 9V/cm forward and 6 V/cm backwards (figure 1c). The 1 kb fragment could not be run to the same position in the forward and reverse 120° pulsed field gel as in the field inversion gel because the fragments would have run off the end of the gel during the last forward phase. In fact, the darkly staining doublet at around 500 bp which ordinarily would be visible as part of this 1 kb ladder has run off the end of the gel. The relative mobility of the DNA from each gel is also plotted in Figure 5B. The initial and final slopes are identical, and the curves are closely matched.

Application to Sequencing Gels

Single stranded DNAs separated in denaturing gels by conventional electrophoresis also migrate as a function of the log of the size. In this case the small fragments, usually on the order of tens of nucleotides are widely spaced, while fragments of more than 500 nucleotides are barely separated. We have previously reported an effect of field switching on the mobility of single stranded DNA in denaturing gels (21). Having now demonstrated that the voltage dependent migration of small double stranded DNA fragments in pulsed field gels can be exploited to reduce the large spacing between the smallest fragments, we sought to determine whether application of these principles could improve the separation of small single stranded fragments.

Figure 6 shows the relative mobility of single stranded fragments in the size range from 50 to 450 nucleotides separated in a denaturing gel by conventional electrophoresis. The region of the curve between 50 and 150 nucleotides is nearly linear. The slope changes above 200 nucleotides, indicating the decrease in resolution. Figure 6 also shows the mobility of the same DNA fragments separated by field inversion electrophoresis using a forward voltage of 1400 V and a reverse voltage of 1000 V. The lower portion of this curve (50 to 100 nucleotides) shows identical mobility to that obtained with conventional electrophoresis. Gels which have been run a shorter time, in which the first 50 nucleotides remain in the gel, also show identical mobility for sizes below 50 nucleotides between conventional and asymmetric-voltage FIGE (not shown). This contrasts to the case for double-stranded DNAs seen above in which the mobility curves differed significantly over the entire size range being separated. However, the portion of this curve corresponding to the larger fragments is clearly affected by the switching, with improved resolution resulting from the asymmetric-voltage FIGE. With these switching conditions, the greatest difference in slope between the two curves is observed for the size range of 150 to 250 nucleotides. The asymmetric-voltage field inversion gel also shows a slight improvement in resolution for the largest fragments on the gels (>350 nucleotides). Comparing the nucleotide sequence of the DNAs separated with the conventional and the field inversion sequencing gels confirmed that no reversal of the size/mobility relationship occurred using these field inversion conditions. We have not been able to similarly improve resolution

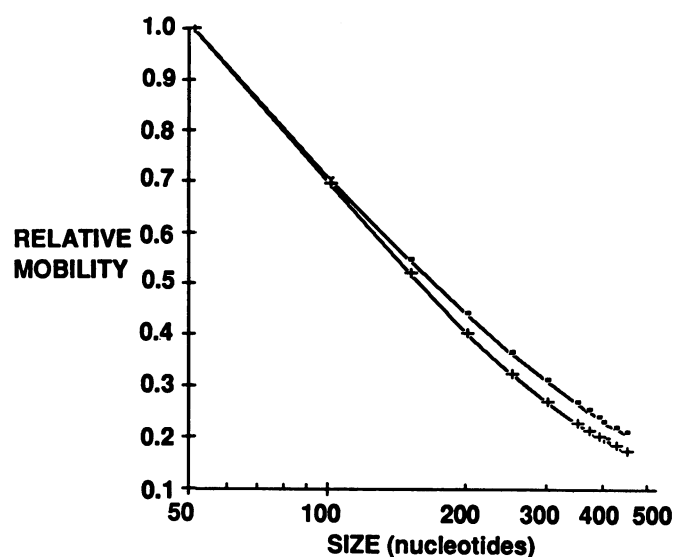


Figure 6. Improved Resolution in Asymmetric-Voltage Field Inversion Sequencing Gels. The mobility of single stranded DNA fragments in a conventional (.) and asymmetric-voltage field inversion sequencing gel (+) is plotted relative to that of a 50 nucleotide fragment. The conventional sequencing gel was run using a constant field of 2800 V and a run duration of 4 hr. The field inversion gel was run for 20 hr using a forward field of 1400 V, 20 msec duration and a reverse field of 1000 V, 2 msec duration. The mobility of the 50 nucleotide fragment was 12.2 cm/hr for the conventional gel, and 3.94 cm/hr for the field inversion gel.

of single stranded DNAs using constant voltage field inversion electrophoresis (not shown). Thus, while figure 6 indicates that asymmetric-voltage field inversion may prove useful in DNA sequencing, our current results with single-stranded DNAs do not yet offer a clear picture of the mechanism or optimal conditions for such separations. Further examination of these issues must await the availability of appropriate high-voltage switching hardware.

DISCUSSION

Unlike other modes of pulsed field gel electrophoresis which use alternating fields of equal magnitude and duration, field inversion requires asymmetry between the alternating fields in either voltage or time. It is well established that the effect of changes in the voltage gradient on the mobility of DNA fragments are not uniform with respect to size. McDonnell et al. have shown that the mobility of fragments less than a few kb is relatively unaffected by variations in the electric field from 0.5 to 10 V/cm. However, there is a progressive increase in sensitivity to voltage as fragment size increased up to 50 kb (3). Because the pulsed field mobility of 1 kb to 50 kb DNA fragments is dependent on the voltage, the migration induced by asymmetric-voltage FIGE differs from that of constant voltage forms of PFG electrophoresis. The difference is valuable because it permits a greater size range of DNA fragments to be resolved on a single gel.

The original concept of an 'effective switch time', which described the pulsed field mobility of larger DNA fragments as the simple product of the voltage and switch interval (5,10) clearly does not apply to these separations. A modified form of this equation was recently derived from a detailed examination of field inversion electrophoresis (22). We have run 1 kb to 50 kb

fragments in 1% gels using switch intervals calculated based on the equation of Heller and Pohl (22) and find similar differences in mobility to those shown above, still indicating an absolute dependence of mobility on voltage (not shown). Pulsed field separations of smaller molecules as described here reflect the combined effects of conventional and pulsed field electrophoresis. Thus the pulsed field behavior of these molecules would not be expected to mimic that seen for much larger molecules for which the contribution of conventional electrophoresis to the separation is negligible.

The final pattern of migration in asymmetric-voltage field inversion electrophoresis of 1–50 kb fragments reflects the difference between the forward and reverse mobilities. The mobilities we report are averages, reflecting net migration over the entire run of the gel. Holzwarth et al. (23) have measured the instantaneous velocity of DNA molecules in agarose gels after changes in field strength and orientation. They showed that the differences in instantaneous velocity are even greater for molecules of different size than are the time-averaged mobilities. Gel conditions which are specifically chosen to make use of the size dependent differences in instantaneous velocity should offer even greater resolution than that which we have demonstrated.

Few direct comparisons of the effectiveness of different modes of pulsed field electrophoresis have been reported. Lasker et al. observed differences in mobility between symmetric-voltage field inversion and the OFAGE configuration of pulsed field electrophoresis for larger DNAs (24). They report co-migration of particular pairs of *Candida* chromosomes during FIGE which are resolved with OFAGE. This is consistent with the differences in the mobility curves generated by the two techniques. In the separation of small fragments we do not see a large difference in the mobility curves generated by symmetric-voltage field inversion and 120° pulsed field gels (fig. 2 and ref. 14). Olson has pointed out the size dependent nature of the drag experienced by molecules which must reorient through angles other than 180° (25). The smaller cross section of fragments between 1 kb and 50 kb may reduce the effective difference between FIGE and other modes of pulsed field electrophoresis for separation of these molecules.

We were eager to observe the behavior of single stranded DNAs subjected to field inversion electrophoresis because, like our work with the pulsed field separation of smaller double stranded fragments, the goal is to extend separation into a greater size range for molecules which ordinarily co-migrate. In addition, like the separation of small double stranded fragments, a large component of the separation achieved with pulsed field sequencing gels will be due to conventional electrophoretic migration. The results suggest that pulsed fields will be valuable in extending the useful range of data collection from sequencing gels. Field inversion is likely to be the favored method of achieving field reorientation in sequencing gels for its simplicity of implementation. We would expect the final migration of the single stranded fragments in field inversion electrophoresis also to reflect the mobility in the separate pulsing conditions used for the forward and reverse fields. Therefore, measurements of single-stranded fragments separated using pulsed fields of different voltage and duration should furnish the data necessary to predict the final FI mobility. We have not been able to carry out such experiments in any detail due to limitations of the existing hardware, which does not permit sufficiently fast high-voltage switching.

Plots of field inversion-induced migration are often quite

complicated, showing multi-phasic relationships between size and mobility (22). The single-stranded migration data we have collected represents just a portion of the mobility curve (the smallest fragments having been run off the end of the gel). Thus, while the curve for field inversion-induced migration for single stranded DNA shown in figure 6 is relatively simple, we do not assume such a simple relationship between size and migration extends beyond the regions of the curve shown. Specifically, it is possible, even expected, that size/mobility reversals would occur outside of the size range we examined when using the electrophoresis conditions we have employed. It is important to note, that reversals of migration and size will not necessarily be detected by the use of size markers of uniform spacing, i.e. 'ladders'.

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